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Simian Virus 40 T-Antigen-Related Cell Surface Antigen: Serological Demonstration on Simian Virus 40-Transformed Monolayer Cells In Situ

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Simian virus 40 (SV40)-transformed monolayer cells were analyzed in situ by indirect immunofluorescence microscopy for the postulated cell surface location of SV40 T-antigen-related molecules. With antisera prepared against purified, sodium dodecyl sulfate-denatured SV40 T-antigen, positive surface staining was obtained when the cells had been treated with formaldehyde before immunofluorescence analysis. In contrast, living SV40-transformed cells analyzed in monolayer were surface fluorescence negative. The fixation procedure developed in this study combined with a double staining immunofluorescence technique allowed the simultaneous analysis of the same cells for the expression of both SV40 T-antigen-related surface antigen and nuclear T-antigen. The localization of SV40 T-antigen-related surface antigen on the outer surface of the plasma membrane of formaldehyde-fixed SV40-transformed cells was demonstrated directly by the protein A-mediated binding of *Staphylococcus aureus* bacteria on formaldehyde-fixed SV40-transformed cells precoated with antiserum against sodium dodecyl sulfate-denatured T-antigen. Both cell surface staining and *S. aureus* binding were found to be highly specific for SV40 T-antigen-related binding sites. These results indicate that T-antigen-related molecules in a cryptic form are located on the surface of SV40-transformed monolayer cells and can be detected in situ after modification of the cell surface architecture.

The simian virus 40 (SV40)-specific antigens T (tumor antigen, T-Ag), U, and TSTA (tumor-specific transplantation antigen) and little t are encoded by the early region of the SV40 genome and are expressed in both SV40-infected and SV40-transformed cells as well as in tumors induced by SV40 (for review, see reference 37). The nuclear antigens T and U are identified by their reaction with antisera from animals bearing SV40-induced tumors (SV40 tumor sera) (22, 28), whereas TSTA is defined by a specific tumor rejection response in animals immunized with SV40 virus or with SV40 tumor cells (6, 17). T-lymphocytes obtained from such animals kill SV40-transformed cells in vitro (14, 35), and an in vivo involvement of T-cells has been demonstrated by the inability of T-cell-depleted animals to develop anti-SV40 tumor immunity (34). Therefore, TSTA is recognized both in vivo and in vitro by host immunocompetent cells and, thus, should be located on the surface of SV40 tumor cells.

Although nuclear T-Ag and TSTA are defined by different immunological criteria, previous experiments have demonstrated a close molecular relationship between both antigens: (i) nuclei isolated from SV40-transformed mouse cells protect susceptible mice against transplantation of SV40 tumors (1, 30), (ii) T and TSTA copurify through different steps of purification (4), and (iii) purified T-Ag has been shown to induce SV40-specific tumor transplantation resistance in mice (5). These data imply that either T-Ag itself or a molecule(s) closely related to it is located on the surface of SV40-transformed cells, and there it is involved in the formation of SV40 TSTA.

We recently reported the surface location of SV40-specific proteins on HeLa cells infected with the nondefective adenovirus type 2-SV40 hybrid viruses Ad2$^+$ND1 and Ad2$^+$ND2 (8, 9). These cells express SV40 U-antigen and SV40 TSTA, but are T-Ag negative (15, 22, 23). The SV40-specific proteins encoded by Ad2$^+$ND1 and Ad2$^+$ND2, which probably carry the antigenic determinants for U and TSTA, have been identified and were shown to correspond to the COOH-terminal part of SV40 T-Ag (25). During the course of infection, SV40-specific hybrid virus proteins accumulate in the plasma membranes of the infected cells (10, 11, 15), supporting the assumption that these proteins are involved in the formation of SV40 TSTA. Analysis of living Ad2$^+$ND1- or Ad2$^+$ND2-infected cells

in monolayer by immunofluorescence microscopy for cell surface location of these proteins with sera from SV40 tumor-bearing animals, as well as with antisera prepared against sodium dodecyl sulfate (SDS)-denatured, gel-purified SV40 T-Ag, however, failed to give a positive result (8, 9). SV40-specific surface fluorescence, on the other hand, was obtained when these cells were fixed with formaldehyde before immunofluorescence analysis and then analyzed with rabbit anti-SDS T-sera (8, 9). Similarly, when living SV40-transformed mouse 3T3 cells (SV3T3 cells) were analyzed in monolayer for SV40-specific cell surface fluorescence, positive surface staining could not be obtained. The same cells, however, became surface fluorescence positive when treated with formaldehyde before immunofluorescence analysis (W. Deppert and R. Henning, Cold Spring Harbor Symp. Quant. Biol., in press). Using a different approach, Lanford and Butel (21) and Soule et al. (33) detected the presence of T-Ag-related antigens on the surface of SV40-infected or -transformed cells. Also in these experiments, T-Ag-related surface antigens were not demonstrated on untreated living cells in monolayer, but only on cells analyzed in suspension after removal from the culture dish with EDTA. Altogether these data suggest that SV40 T-Ag-related surface antigen(s) on SV40-transformed monolayer cells may be cryptic and only detectable upon alteration of the cell surface architecture.

In this study we further improved the formaldehyde fixation procedure for the in situ analysis of SV40 T-Ag-related surface antigen on SV40-transformed monolayer cells. By using this fixation procedure, it was possible to demonstrate that SV40 T-Ag-related surface antigens become detectable in situ on the surface of all SV40-transformed cells analyzed so far. By combining this fixation procedure with a double-labeling immunofluorescence technique, SV40 T-Ag-related surface antigen and nuclear T-Ag could be visualized simultaneously on the same cells.

MATERIALS AND METHODS

Cells. Mouse BALB/c fibroblasts (3T3 cells), SV40-transformed 3T3 cells (SV3T3 cells and SV3T3, clone SV101), polyoma-transformed 3T3 cells (Py3T3), mouse BALB/c SV40 tumor cells (VLM cells [38], kindly provided by M. and S. Tevethia, Pennsylvania State University, Hershey, Pa.), and cells of the hamster tumor cell line H 65/90 B (kindly provided by V. Defendi, New York University, New York, N.Y.) as well as HeLa cells were grown in Dulbecco-modified Eagle medium supplemented with 10% calf serum.

African green monkey kidney cells (CV1 cells), SV40-transformed CV1 cells (C2 cells, kindly provided by G. Sauer, German Cancer Research Institute, Heidelberg, West Germany), human fibroblasts (WI 38 cells, kindly provided by M. Osborn, Max Planck Institute, Göttingen, West Germany), SV40-transformed human fibroblasts (SV80 cells), secondary rat embryo fibroblasts (GIBCO Laboratories, Grand Island, N.Y.), and rat embryo fibroblasts transformed by the temperature-sensitive SV40 *A* gene mutant *tsA* 28.3 (*tsA* 28.3 cells [27], kindly provided by M. Osborn) were grown in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum.

Sera. (i) Hamster SV40 tumor sera. Hamster SV40 tumor sera were obtained from outbred Syrian gold hamsters 3 to 4 weeks after injection with 10^5 to 10^6 H 65/90 B cells. These hamster SV40 tumor sera were used individually or pooled after screening for high titers against T-Ag by immunofluorescence microscopy (28) (pools 1 and 2).

(ii) Mouse SV40 tumor sera. SV40 tumor sera were produced in 10- to 12-week-old male BALB/c mice by subcutaneous inoculation of 5×10^5 SV40-transformed BALB/c mouse cells (VLM). Sera were collected and pooled 6 weeks after inoculation at a tumor size of approximately 1 to 2 cm.

(iii) Anti-SDS T-sera. Anti-SDS T-sera were prepared as described previously (8, 9). Briefly, T-Ag was extracted from either H 65/90 B cells or SV80 cells and purified by immunoprecipitation by the method of Schwyzer (31) and subsequent SDS-gel electrophoresis. For immunization with T-Ag, the following procedure was employed: T-Ag from H 65/90 B cells (rabbit anti-SDS T-serum 1) or SV80 cells (rabbit anti-SDS T-serum 2) was electrophoretically eluted from the preparative SDS-polyacrylamide gels and dialyzed against 0.1% SDS in water. Portions (0.5 ml, ~40 μg) were mixed with Freund complete adjuvant and injected subcutaneously into a rabbit. Animals received three injections at 3-week intervals before they were bled. The production of antisera against purified, SDS-denatured SV40 T-Ag by essentially similar procedures has been described by other investigators (3, 20, 21).

(iv) Rabbit anti-mouse cell surface serum. mKSA-Asc cells were grown in Joklik-modified minimal essential medium for suspension culture (F-13; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% calf serum. Cells (5×10^7) were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS), and fixed for 10 min in 10 ml of 3.7% formaldehyde in PBS. After fixation, the cells were washed five times with PBS and resuspended in 500 μl of PBS. A rabbit received three intravenous injections at 4-week intervals before the serum was collected.

(v) Rabbit anti-actin-serum. Actin was extracted from chicken muscle (2), and it was purified by preparative SDS-gel electrophoresis. Actin was eluted from gels and lyophilized. Six 0.5-mg amounts were injected subcutaneously during 3 weeks. Ten days after the last injection the rabbits were bled. The sera were titrated both by Ouchterlony double diffusion (titer, 1:32) and by indirect immunofluorescence (titer, 1:160) on methanol-acetone-fixed 3T3 cells.

Absorption of sera. Before determination of T-

and U-antibody titers and before analysis of cells for SV40-specific cell surface fluorescence, sera from tumor-bearing animals and anti-SDS T-sera were absorbed on methanol-fixed mouse 3T3 cells. Confluent monolayers of 3T3 cells grown on plastic petri dishes (9 cm in diameter) were washed twice with PBS and then fixed with methanol for 10 min at room temperature. After air drying, the cells were rinsed with PBS, and 1-ml aliquots of the sera diluted 1:10 with PBS were added to each plate. The sera were absorbed three times for 12 h at 4°C.

Secondary antibodies (fluorescein isothiocyanate [FITC]- or tetramethylrhodamine isothiocyanate-labeled immunoglobulin prepared against immunoglobulin of the first antibody) were adjusted to a protein concentration of about 0.5 mg/ml and absorbed as described above. After absorption, all sera and second antibodies were cleared of particulate material by a 4-min centrifugation at 8,000 × *g* in an Eppendorf centrifuge.

Second antibodies. FITC-labeled or tetramethylrhodamine isothiocyanate-labeled immunoglobulin prepared against immunoglobulin of the first antibody were from the following sources: (i) goat immunoglobulin, fluorescein labeled, prepared against hamster immunoglobulin (Cappel Laboratories, Inc., Cochranville, Pa.); (ii) fluorescein- or rhodamine-labeled rabbit immunoglobulin, prepared against mouse immunoglobulin (Nordic/Byk Mallinckrodt, Dietzenbach, West Germany); (iii) fluorescein-labeled sheep immunoglobulin, prepared against rabbit immunoglobulin (Miles Laboratories, Inc. Frankfurt, West Germany).

Immunofluorescence microscopy. Cells to be analyzed in immunofluorescence microscopy were grown on glass cover slips (12 mm in diameter) in Dulbecco-modified Eagle medium supplemented with either 10% calf serum or 10% fetal calf serum, depending on the cell line analyzed.

Analysis of T- and U-antibody titers. Antibody titers against SV40 T-Ag were determined on methanol–acetone-fixed SV101 cells as described elsewhere (7, 9). For determination of U-antibody titers in SV101 cells, T-Ag was heat inactivated before analysis as described previously (7, 9).

Cell surface staining and double staining immunofluorescence microscopy. Cells on cover slips were fixed for 1 h in 3.7% formaldehyde (freshly prepared from paraformaldehyde; Serva, Heidelberg, West Germany) in PBS, containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The temperature during the fixation process was kept between 0 and 2°C to prevent antibody permeability of the cells (see Results). After fixation, the cells were washed with PBS, and the first antibody, serum from SV40 tumor-bearing animals or anti-SDS T-sera, diluted 1:10 with PBS, was added. The cover slips were incubated for 45 min at 37°C, washed with PBS, and incubated with fluorescein-labeled immunoglobulin prepared against immunoglobulin of the first antibody for another 45 min at 37°C. After another series of washes with PBS, the cover slips were either mounted directly with Elvanol on microscope slides or processed for double staining immunofluorescence microscopy.

When processed for further analysis of intracellular structures, after incubation with first and second antibodies, the cover slips were washed with PBS followed by a 30-s treatment with methanol (room temperature) and then permeabilized by treatment with acetone for 30 min at room temperature. After air drying, the cells were incubated with either mouse SV40 tumor serum (for visualization of nuclear T-Ag) or rabbit anti-actin antibody (for visualization of actin microfilaments) for 45 min at 37°C. The cells were then washed with PBS and incubated with FITC- or tetramethylrhodamine isothiocyanate-labeled immunoglobulin prepared against immunoglobulin of the first antibody. After another series of washes with PBS, the cover slips were mounted with Elvanol on microscope slides.

The most critical step of this procedure was the formaldehyde fixation. Success in terms of antibody impermeability for analysis of surface fluorescence and in terms of the ability to permeabilize the formaldehyde-fixed cells by additional treatment with organic solvents strongly depended on the brand of paraformaldehyde used. Out of several brands tested, best results were obtained with paraformaldehyde from Serva, Heidelberg, West Germany, although with this brand some batches were found to induce antibody permeability to the cells, too.

For immunofluorescence analysis cells were viewed with a Zeiss microscope (Carl Zeiss, Oberkochen, West Germany) equipped with epifluorescent illumination. Pictures were taken with Planapo ×40 and ×63 oil-immersion objectives.

Scanning electron microscopy of the binding of *Staphylococcus aureus* to the cell surface. 3T3 or SV3T3 cells were grown on cover slips and after rinsing with PBS were fixed for 1 h with 3.7% formaldehyde in PBS at 0 to 2°C, washed with PBS, and used without air drying. Formaldehyde-fixed cells were incubated with rabbit anti-SDS T-serum or normal rabbit serum at 0 to 2°C for 60 min. The cover slips were then rinsed several times in PBS and incubated in a 1% suspension of *S. aureus* in PBS for 2 h at 4°C. A similar procedure was described by Ghetie et al. (13). Washing was performed to remove excess *S. aureus* from the cover slips under careful control by light microscopy. *S. aureus* (strain Cowan I) was grown exactly as described by Kessler (16) and fixed with 1% formaldehyde for 30 min at room temperature. *S. aureus* was inactivated by heating at 80°C for 30 min.

To prepare the samples for scanning electron microscopy, cells were fixed with 1% glutaraldehyde in PBS for 5 min at room temperature. After fixation, the cells were thoroughly washed with water and dehydrated in acidified 2,2'-dimethoxypropane (26). Subsequently, the samples were dried by critical point drying and sputtered with 10.0 nm of gold. The cells were viewed in a Phillips scanning electron microscope (PSEM 500). Scanning electron microscopy was kindly performed by Dieter Frösch, University of Ulm.

Papain treatment of living SV3T3 cells in monolayer. SV3T3 cells were grown on cover slips in 3-cm petri dishes as described above, washed with PBS, and incubated as monolayer with papain immobilized on carboxymethyl cellulose (Sigma) at room

temperature (0.2 BAEE [α-*N*-benzoyl-arginine ethyl ester] units per ml of PBS, corresponding to a concentration of ~10 μg of papain type III per ml of PBS). After the times indicated (see Results), cover slips were removed from the petri dish, washed with PBS, and processed for cell surface staining as described above. Experimental conditions were chosen in which the least possible number of cells was removed from the culture dish by the action of the protease. Typically, no more than 10 to 20% rounded up during a 20-min exposure to papain.

RESULTS

Antisera. Two types of antisera were used to analyze SV40-transformed cells for the expression of SV40 T-Ag-related surface antigen: (i) sera from SV40 tumor-bearing animals and (ii) antisera prepared against SDS-denatured T-Ag, purified electrophoretically from immunoprecipitates of nonionic detergent (Nonidet P-40) extracts prepared from SV40-transformed hamster (H 65/90 B) or human (SV80) cells by the method of Schwyzer (31). Analysis of the electrophoretically eluted SV40 T-Ag on SDS-gels or on gels containing SDS and urea showed that in both gel systems the eluted protein migrated as a single protein band (data not shown). This result greatly reduced the possibility of a host protein being coprecipitated and contaminating the T-Ag preparation. One therefore can conclude that the only protein species serving as antigen was SV40 T-Ag.

Both types of sera were first characterized by determination of their T- and U-antibody titers on SV40-transformed mouse cells (SV3T3 cells). Table 1 shows that all sera listed exhibited high titers both for T- and U-antibodies. With the exception of hamster pool 2 serum, which had a fourfold-higher titer against T-Ag, the T- and U-antibody titers of these sera were similar. The SV40 specificity of these sera was further analyzed by indirect immunoprecipitation of Nonidet P-40 detergent extracts (31) of radioactively ([^{3}H]leucine, [^{35}S]methionine) labeled SV40-transformed cells of different species followed by SDS-polyacrylamide gel electrophoresis. All antisera listed in Table 1 specifically precipitated the 94K large T-Ag, the 17K little t-antigen, and varying amounts of the host-coded 53K nonviral T-Ag (19), whereas the corresponding nonimmune sera did not precipitate these proteins (Deppert and Henning, in press; unpublished data). In addition, all antisera specifically precipitated the SV40-specific 42K and 56K proteins from extracts of Ad2$^+$ND2-infected HeLa cells (9).

Analysis of SV40-transformed cells for T-Ag-related surface antigen. Living SV40-transformed cells in monolayer were analyzed for SV40 T-Ag-related surface antigen (surface T) by immunofluorescence microscopy with the sera listed in Table 1. In none of these experiments could positive cell surface staining be observed (for a representative example, see Fig. 1a). Recently, however, two different techniques have been described for the analysis of SV40-transformed monolayer cells for surface T which resulted in positive cell surface staining. (i) Lanford and Butel (21) and Soule et al. (33) have analyzed single-cell suspensions of SV40-transformed cells grown in monolayer after removal from the culture dishes by treatment with EDTA. The suspended cells were then surface fluorescence positive, both with rabbit anti-SDS T-serum and with SV40 tumor sera. (ii) With a different approach, we have developed a method for analyzing the in situ expression of SV40 surface T on SV40-transformed monolayer cells. Such a method seemed desirable because most of the SV40-transformed cell lines in use grow only as monolayer cells and not in suspension culture. In our initial attempts, we have pretreated SV40-transformed cells in monolayer with 3.7% formaldehyde in PBS at 0°C followed by air drying before immunofluorescence analysis and obtained positive surface fluorescence with rabbit anti-SDS T-sera, but not with SV40 tumor sera (Deppert and Henning, in press; J. Lange-Mutschler, W. Deppert, K. Hanke, and R. Henning, submitted for publication). Although these data strongly indicated the presence of T-Ag-related molecules also on the surface of adherent SV40-transformed monolayer cells, the in situ analysis of these cells by this method had certain disadvantages. (i) The cell surface fluorescence obtained was weak and could be detected only at dilutions of the rabbit

TABLE 1. *T- and U-antibody titers of sera from SV40 tumor-bearing animals and of anti-SDST-sera*

Serum	Antibody titers[a] on:	
	SV101 cells (T)	Heated SV101 cells (U)
Hamster SV40 tumor serum, pool 1	800	600
Hamster SV40 tumor serum, pool 2	3,200	800
Mouse SV40 tumor serum	3,200	3,200
Rabbit anti-SDS T-serum 1	1,600	1,600
Rabbit anti-SDS T-serum 2	3,200	1,600

[a] Antibody titers were determined as described in the text. Titers are given as reciprocal of the last serum dilution at which the staining was still positive. The variation of the titers between separate experiments corresponds to ± one dilution step.

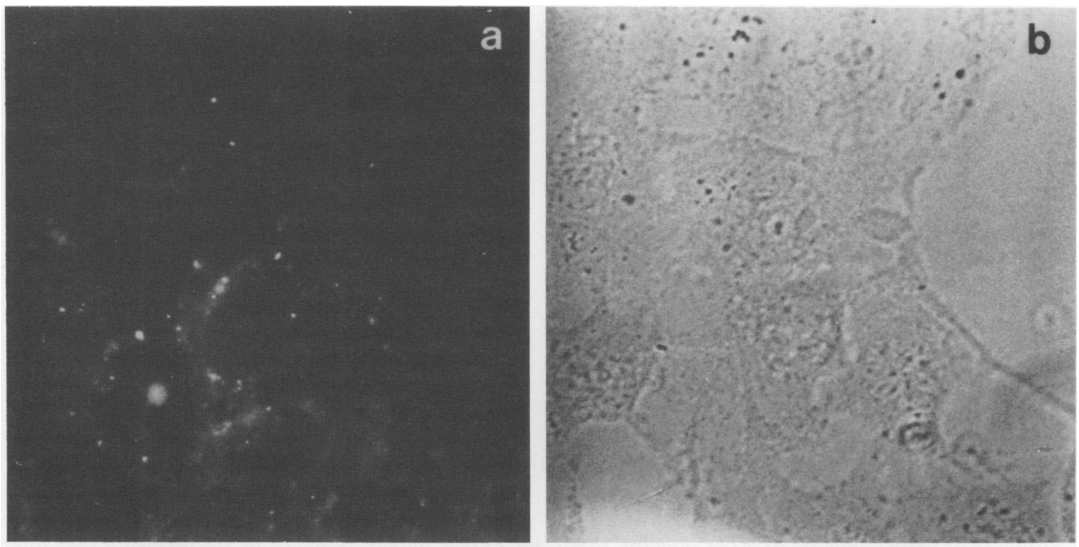

FIG. 1. *Analysis of living SV3T3 monolayer cells in situ for SV40-specific cell surface fluorescence using rabbit anti-SDS T-serum. SV3T3 cells were grown in monolayer on cover slips and, after washing with PBS, incubated with 5 μl of rabbit anti-SDS T-serum, diluted 1:10 in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered Dulbecco-modified Eagle medium followed by FITC-labeled sheep anti-rabbit immunoglobulin as the second antibody. (a) Immunofluorescence microscopy (2.5-min exposure); (b) light microscopy. Magnification is 540-fold.*

anti-SDS T-sera down to 1:20. (ii) Permeabilization of the formaldehyde-fixed and air-dried cells by additional treatment with organic solvents proved to be extremely difficult. Therefore, nuclear T-Ag or cytoplasmic structures (e.g., cytoskeletal structures) could no longer be visualized in these cells. The simultaneous demonstration of both nuclear T-Ag and surface T within the same cells, however, should provide a valuable means for analyzing the expression of both antigens in SV40-transformed cells.

To improve the condition for the in situ analysis of adherent SV40-transformed monolayer cells for both cell surface and intracellular antigens, several variations of the fixation procedure were investigated: (i) formaldehyde concentration (1 to 10%), (ii) time (5 min to 2 h), and (iii) temperature of the fixation process (0°C, room temperature [20°C]). Optimal results in terms of preservation of cell shape and in terms of positive SV40-specific cell surface staining were obtained when the cells were fixed with 3.7% formaldehyde (freshly prepared from paraformaldehyde) at 0°C for 1 h in the presence of 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. An important advantage of this new fixation procedure was that the cells were not permeable for antibodies but still could be permeabilized by additional treatment with organic solvents. This was tested by staining SV3T3 cells, fixed by the formaldehyde treatment described above, with rabbit antiserum to actin. Figure 2a demonstrates that under these conditions no actin-specific fluorescence was obtained, indicating that the actin antibodies were not able to permeate the plasma membrane. If, however, formaldehyde-fixed SV3T3 cells were additionally treated with methanol-acetone (see Materials and Methods) before incubation with the rabbit anti-actin serum, a brilliant staining of the actin microfilament system was observed (Fig. 2b). It is important to note that the temperature during fixation had to be kept at 0°C to find less than 5% of the fixed cells permeable for antibody. If the temperature during fixation was raised only slightly (>4°C), as much as 50% of the cells showed positive actin fluorescence without further permeabilization with organic solvents.

By using this procedure 3T3, SV3T3 and VLM cells were analyzed with rabbit anti-SDS T-sera for the presence of T-Ag-related molecules on the cell surface. Figure 3a shows that 3T3 cells were surface fluorescence negative, whereas both SV40-transformed mouse cell lines exhibited positive surface fluorescence (Fig. 3b and c). The titer for positive surface staining of SV3T3 cells or VLM cells was 1:60 for both rabbit anti-SDS T-sera listed in Table 1. As in our previous studies, analysis of formaldehyde-fixed, SV40-transformed cells with the SV40 tumor sera listed in Table 1 did not result in positive surface staining.

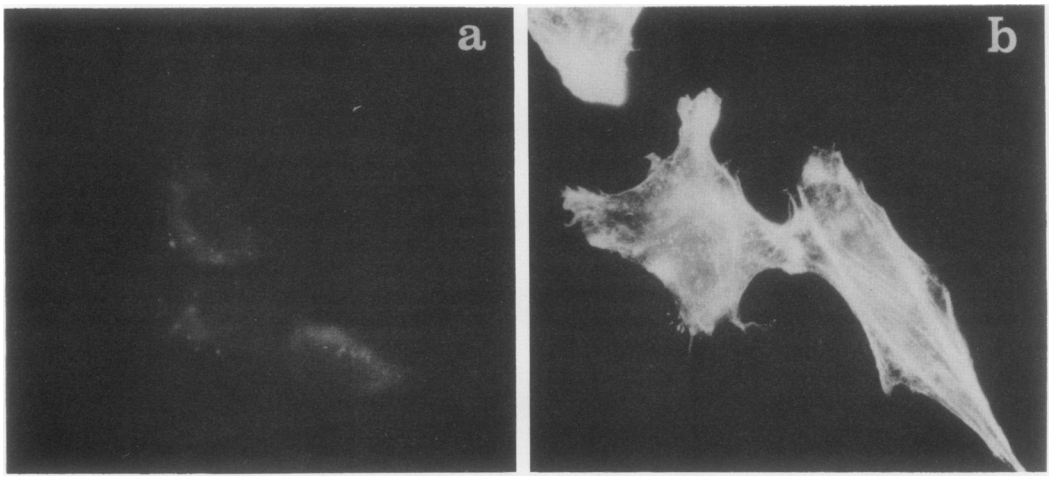

FIG. 2. *Analysis of formaldehyde-fixed SV3T3 cells with rabbit anti-actin antibody. SV3T3 cells grown on cover slips were fixed with 3.7% formaldehyde in PBS for 1 h at 0°C and were (a) stained directly with rabbit antibody to actin followed by FITC-labeled sheep anti-rabbit immunoglobulin or (b) permeabilized after formaldehyde fixation by treatment with methanol and acetone and then stained with anti-actin antibody as for (a). Exposure times were 2.5 min (a) or 5 s (b). Magnification is 450-fold.*

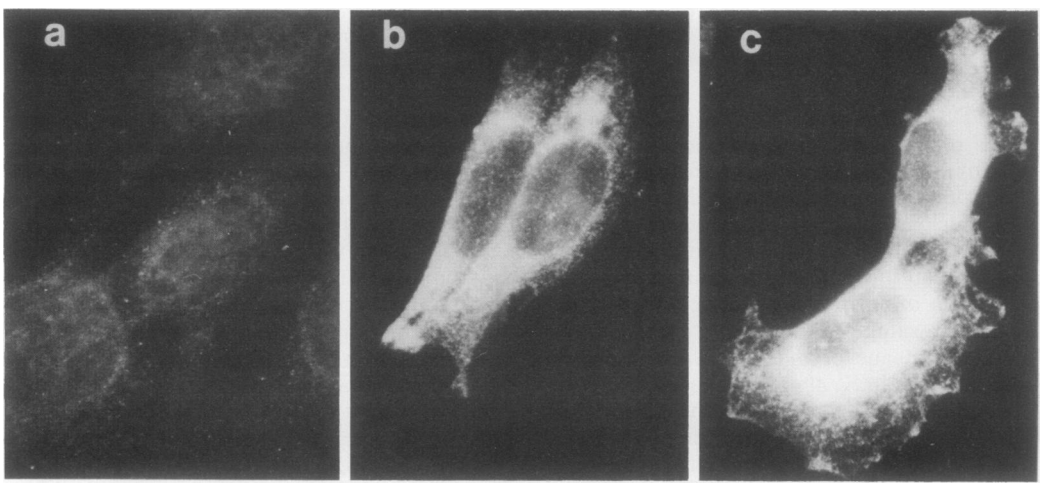

FIG. 3. *Analysis of formaldehyde-fixed 3T3 cells, SV3T3, and VLM cells for SV40-specific cell surface fluorescence with rabbit anti-SDS T-serum. 3T3 cells, SV3T3 cells, and VLM cells grown on cover slips were fixed with formaldehyde and processed for immunofluorescence microscopy. Rabbit anti-SDS T-serum 1, at a 1:10 dilution with PBS, was used as first antibody, and FITC-labeled sheep anti-rabbit immunoglobulin was used as second antibody. Staining with rabbit anti-SDS T-serum 2 resulted in identical cell surface fluorescence. Exposure times were 2.5 min (a) and 30 s (b and c). Magnification is 400-fold.*

The morphology of surface staining as shown in Fig. 3 is different from the typical surface fluorescence of living cells in suspension. The fluorescence intensity is not enhanced at the rims and the protrusions of the cells, but is distributed in patches over the whole surface of the cells. Since the surface fluorescence is weak and the plasma membrane above the nuclei is out of the focal plane, the surface area above the nuclear region seems to be devoid of stain. By testing formaldehyde-fixed SV3T3 cells with rabbit anti-mouse cell surface antiserum (prepared against formaldehyde-fixed mKSA cells), a cell surface staining similar to that in Fig. 3b and c was obtained (data not shown). One therefore can assume that the morphology of the SV40-specific surface fluorescence seen in Fig. 3b and c reflects a typical cell surface staining on formaldehyde-fixed monolayer cells.

Cell surface staining similar to that shown in

Fig. 3 was obtained when other SV40-transformed cells of different species origin were analyzed (SV40-transformed monkey cells [line C2], human cells [SV80], and rat cells [*tsA* 28.3]), but not on their untransformed counterparts (CVI cells, WI 38 cells, rat embryo fibroblasts) (data not shown), documenting the SV40 specificity of the cell surface staining. The specificity of the cell surface staining on SV40-transformed cells for SV40 T-Ag-related binding sites was further analyzed with rabbit anti-SDS T-serum, which had been absorbed on methanol-fixed SV3T3 cells, i.e., cells exposing nuclear T-Ag. This absorption resulted in a more than 80% reduction of the T- and U-antibody titers in this serum (9). Since this antiserum was prepared against SDS-denatured nuclear T-Ag, anti-T-Ag antibodies in this serum would more likely be directed against the primary structure of T-antigen rather than against its native conformation. Removal of these antibodies by absorption on nuclear T-Ag, therefore, should also remove antibodies which recognize surface T. In accordance with this assumption, the SV3T3-absorbed anti-SDS T-serum no longer was able to stain the cell surface of any SV40-transformed cell analyzed, regardless of species origin. Since the surfaces of human or monkey cells, for example, are antigenically quite different from the surface of mouse cells, this finding is again a strong argument for a SV40-specific cell surface staining with rabbit anti-SDS T-serum. In addition, absorption of the rabbit anti-SDS T-serum on homologous non-SV40-transformed cells, e.g., on HeLa cells for analysis of SV80 cells or on Py3T3 cells for analysis of SV3T3 cells, did not remove the ability of this serum to stain the surface of the SV40-transformed cells. Altogether, these data indicate that the surface fluorescence seen on all SV40-transformed cells tested was not due to a general transformation-induced surface alteration, but rather reflected the expression of SV40 T-Ag-related proteins on the surfaces of these cells.

Analysis of SV40-transformed cells for surface T and nuclear T-Ag by double staining immunofluorescence microscopy. The new fixation procedure enabled us to analyze cells simultaneously for the expression of surface and nuclear antigens by double staining. SV3T3 cells were first fixed with formaldehyde and surface stained with rabbit anti-SDS T-serum followed by FITC-coupled sheep anti-rabbit immunoglobulin (Fig. 4a). Next, the cells were permeabilized by treatment with methanol–acetone and stained for nuclear T-Ag with mouse SV40 tumor serum followed by tetramethylrhodamine isothiocyanate-coupled rabbit anti-mouse immunoglobulin (Fig. 4b). Comparison of Fig. 4a and b shows that all SV3T3 cells exhibiting surface staining were also positive for nuclear T-Ag. However, it should be noted that only a certain percentage of the nuclear T-Ag-positive cells exhibited cell surface staining, whereas others were not or only very weakly surface positive. This observation is also demonstrated in Fig. 4. The arrow in Fig. 4b points to a cell which is brilliantly stained for nuclear T-Ag, but completely negative for SV40 surface T (Fig. 4a, arrow).

By comparing nuclear T-Ag immunofluorescence titers of mouse SV40 tumor serum (see Table 1) on formaldehyde-fixed and then permeabilized SV3T3 cells with the titer of this serum on SV3T3 cells fixed by methanol–acetone alone, no gross titer differences were detected. We therefore assume that (i) the formaldehyde fixation procedure employed did not severely alter the antigenicity of the nuclear T-Ag and (ii) that nuclear T-Ag did not leak from the nuclei during the fixation process. The theoretical argument that nuclear T-Ag might leak out of dead cells either before or during the fixation process and then might precipitate on the cell surface during formaldehyde fixation, thereby causing the SV40-specific cell surface fluorescence seen on formaldehyde-fixed SV40-transformed cells, could be largely excluded by the following experiment. 3T3 and SV3T3 cells were grown in mixed culture on cover slips, fixed with formaldehyde, and stained for surface T and then permeabilized and stained for nuclear T-Ag. If SV40-specific cell surface staining would be due to unspecific binding of exogenous T-Ag to the cell surface, one should expect to find surface staining on both cell types. Figure 5a and b demonstrate that only cells expressing nuclear T-Ag, i.e., SV3T3 cells, showed positive cell surface fluorescence, whereas 3T3 cells were negative for both nuclear and surface staining. No cells were positive for surface fluorescence and simultaneously negative for nuclear T-Ag.

Location of SV40 surface T on the outer surface of the plasma membrane. Analysis of SV40-transformed cells by the formaldehyde fixation procedure employed for expression of surface T resulted in a staining pattern which so far had been described as surface staining only because actin antibodies were not able to permeate the plasma membrane of the fixed cells and nuclear T-Ag could not be visualized in these cells (Fig. 2a and 3a and b). To demonstrate more directly the location of SV40 surface T on the outer surface of the plasma membrane of formaldehyde-fixed SV3T3 cells, we analyzed the binding of *S. aureus* on formaldehyde-fixed

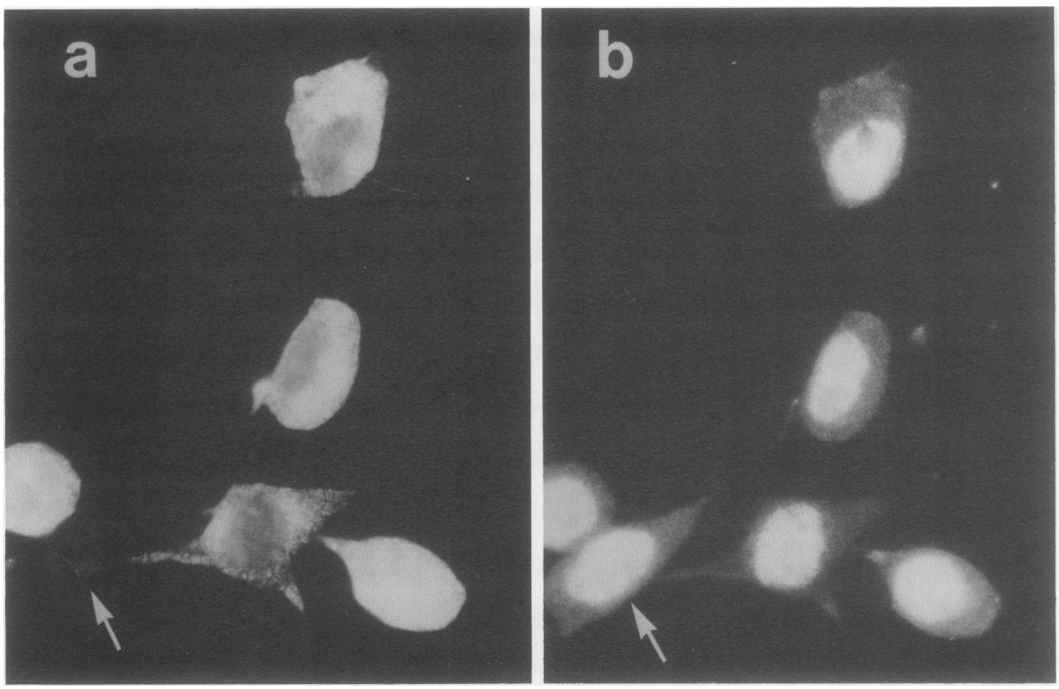

FIG. 4. *Double-staining of SV3T3 cells for T-Ag-related cell surface antigen and for nuclear T-Ag. SV3T3 cells grown on cover slips were fixed with formaldehyde and first stained for cell surface fluorescence with rabbit anti-SDS T-serum followed by FITC-coupled sheep anti-rabbit immunoglobulin. The same cells were then permeabilized with methanol and acetone and stained additionally for nuclear T-Ag with mouse SV40 tumor serum followed by tetramethyl rhodamine isothiocyanate-coupled rabbit anti-mouse immunoglobulin. (a) Fluorescein fluorescence of cell surface staining; (b) rhodamine fluorescence of nuclear T-Ag staining. Arrows point to a cell which is cell surface fluorescence negative (a) but is brilliantly stained for nuclear T-Ag(b). Exposure times were 30 s for (a) and (b). Panel (b) was slightly overexposed to show the contours of the cells. Magnification is 600-fold.*

3T3, Py3T3, or SV3T3 cells which were precoated with either rabbit anti-SDS T-serum or with nonimmune serum. The surface of these bacteria exposes protein A, which has been shown to react with high specificity with the Fc region of several classes of immunoglobulin from different species (18). Because of the size of these bacteria, a specific binding of *S. aureus* on the surface of formaldehyde-fixed SV40-transformed cells precoated with rabbit anti-SDS T-serum would be direct proof that T-antibodies react with molecules on the outer surface of the plasma membrane. As seen in Fig. 6a, *S. aureus* bound only to the surface of formaldehyde-fixed SV3T3 cells precoated with rabbit anti-SDS T-serum, but not to the same cells coated with normal rabbit serum (Fig. 6b). *S. aureus* binding was not observed on control cell lines (3T3, Py3T3) coated with rabbit nonimmune serum or with anti-SDS T-serum or when SV3T3 cells were precoated with hamster SV40 tumor serum. Precoating of living SV3T3 cells with either rabbit anti-SDS T-serum or with SV40 tumor serum also did not induce *S. aureus* binding. All these results demonstrate that the *S. aureus* binding on SV40-transformed cells occurred with the same specificity as positive surface fluorescence in immunofluorescence analysis.

In a different approach to demonstrate the location of SV40 T-Ag-related molecules on the outer surface of the plasma membrane, living SV3T3 cells were treated as an intact monolayer with carboxymethyl cellulose coupled papain for various lengths of time (see Materials and Methods), then fixed with formaldehyde and analyzed for SV40-specific cell surface fluorescence. Figure 7b demonstrates that already after a 10-min pretreatment with papain, SV40-specific cell surface fluorescence was greatly reduced when compared to untreated cells (Fig. 7a) and was virtually abolished after a 20-min papain treatment of the cells (Fig. 7c). Since treatment of living cells with immobilized papain most probably acted only on cell surface proteins, the reduction in SV40-specific surface fluorescence after papain treatment strongly suggests that

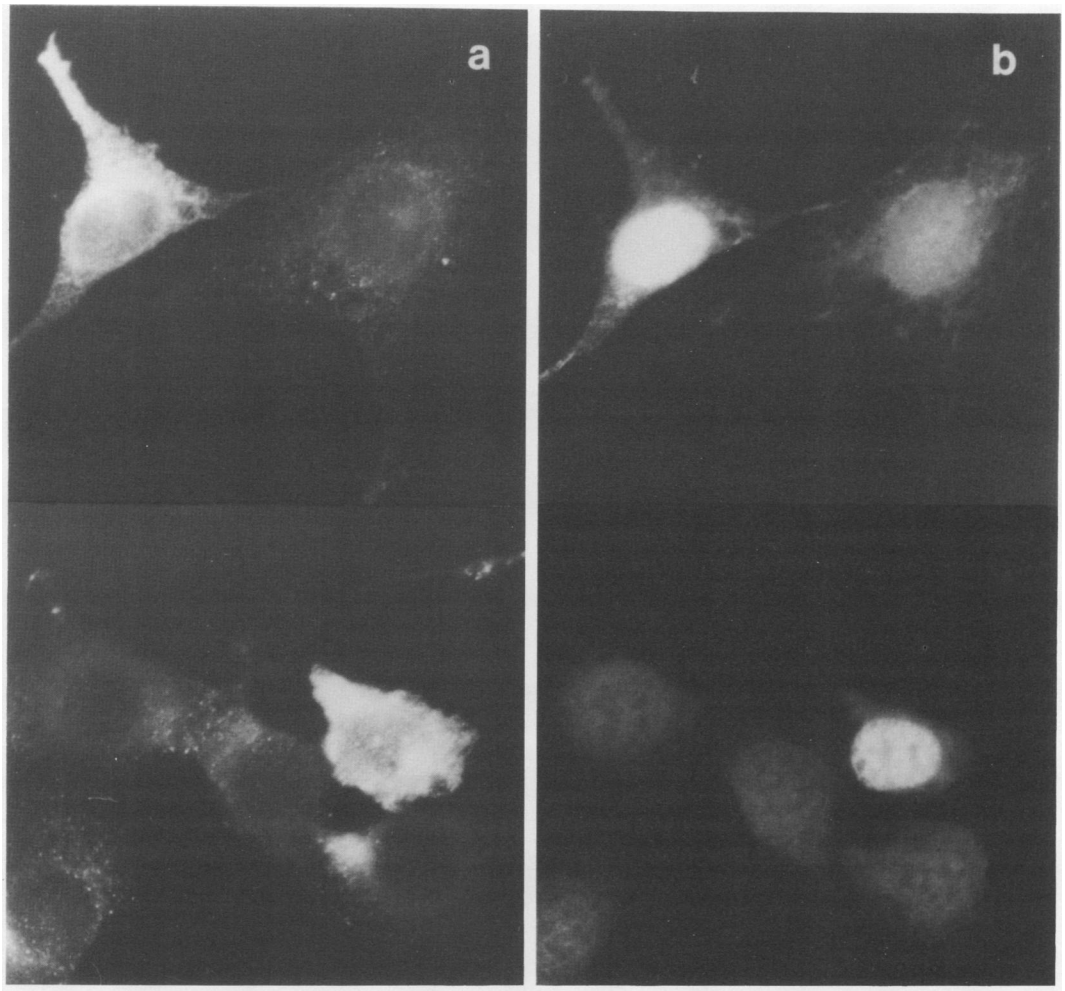

FIG. 5. *Analysis of a mixed culture of 3T3 cells and SV3T3 for T-Ag-related surface antigen and nuclear T-antigen by double staining. 3T3 cells and SV3T3 cells were grown in a mixed culture on cover slips and first stained for cell surface fluorescence with rabbit anti-SDS T-serum followed by FITC-labeled sheep anti-rabbit immunoglobulin. The same cells were then permeabilized with methanol and acetone and stained for nuclear T-Ag with mouse SV40 tumor serum followed by tetramethylrhodamine isothiocyanate-coupled rabbit anti-mouse immunoglobulin. (a) Fluorescein fluorescence of cell surface staining; (b) rhodamine fluorescence of nuclear T-Ag staining. Exposure times in (a) and (b) were 30 s. Magnification is 500-fold.*

SV40 T-Ag-related molecules reacting with rabbit anti-SDS T-serum on formaldehyde-fixed cells are indeed located on the cell surface.

DISCUSSION

Although the presence of T-Ag-related molecules on the surface of SV40-transformed cells has been postulated to explain the TSTA activity of nuclear T-Ag (1, 5, 30), attempts to demonstrate the presence of such molecules on the surface of living SV40-transformed cells in monolayer by serological methods so far have failed (5). Recently, however, SV40-specific cell surface fluorescence was demonstrated on living SV40-transformed cells grown in monolayer, but only after the cells had been removed from the culture dish by treatment with EDTA before immunofluorescence analysis (21, 32). We recently found that T-Ag-related binding sites could be detected by immunofluorescence microscopy also on the surface of SV40-transformed monolayer cells which had been put into suspension mechanically by squirting them off the culture dish with a Pasteur pipette. Furthermore, when living mKSA cells grown in suspen-

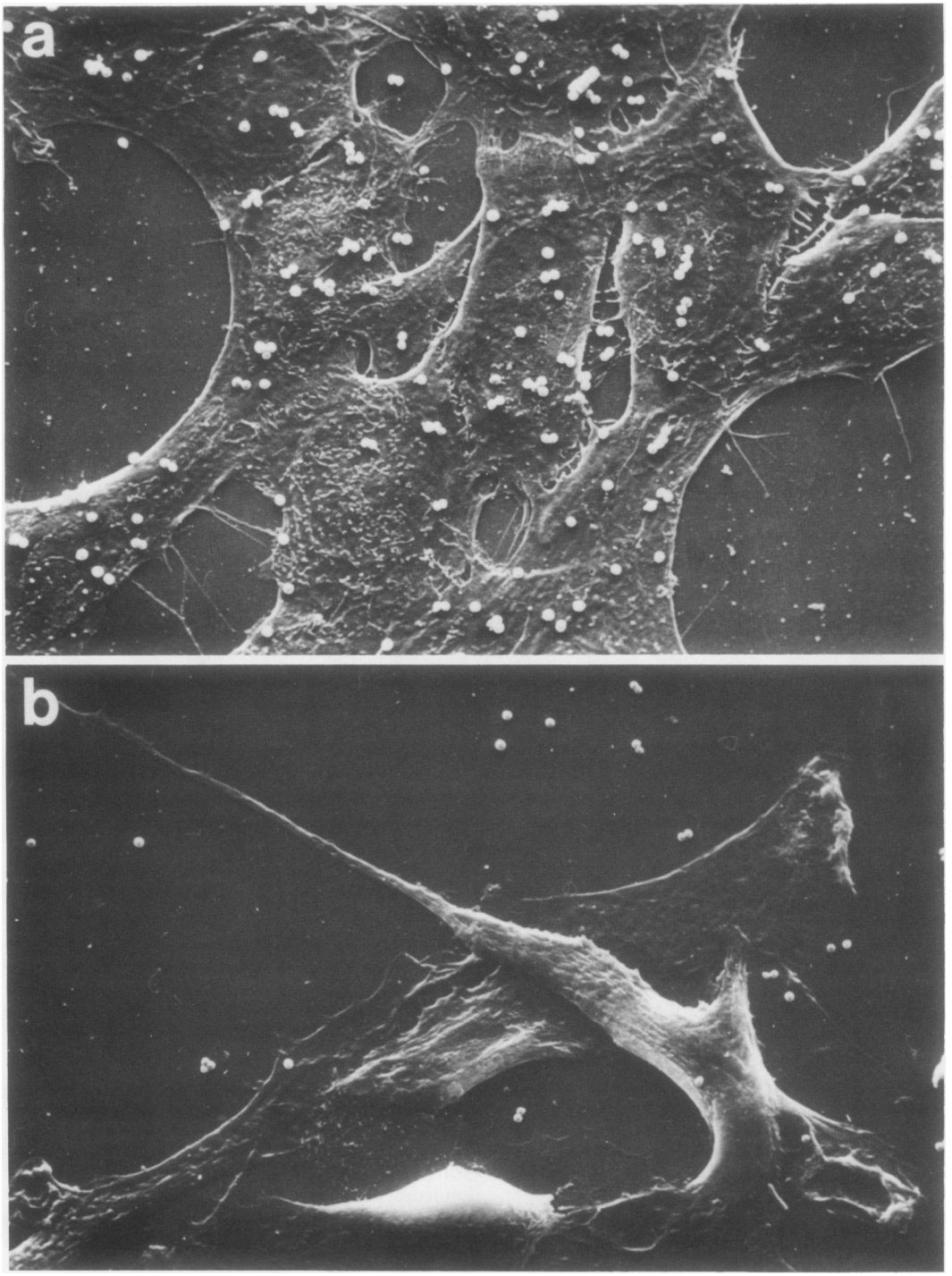

FIG. 6. *Scanning electron micrographs of formaldehyde-fixed SV3T3 cells incubated with rabbit anti-SDS T-serum (a) or normal rabbit serum (b) and S. aureus. (a) SV3T3 cells were fixed with 3.7% formaldehyde in PBS for 5 min and air dried. After rinsing with PBS, the cells were incubated with rabbit anti-SDS T-serum for 60 min at 0 to 2°C and subsequently with a 1% suspension of S. aureus. (b) SV3T3 cells (treated as in a) were incubated with normal rabbit serum (60 min, 0 to 2°C) and subsequently with 1% suspension of S. aureus. Magnification is 1,500-fold.*

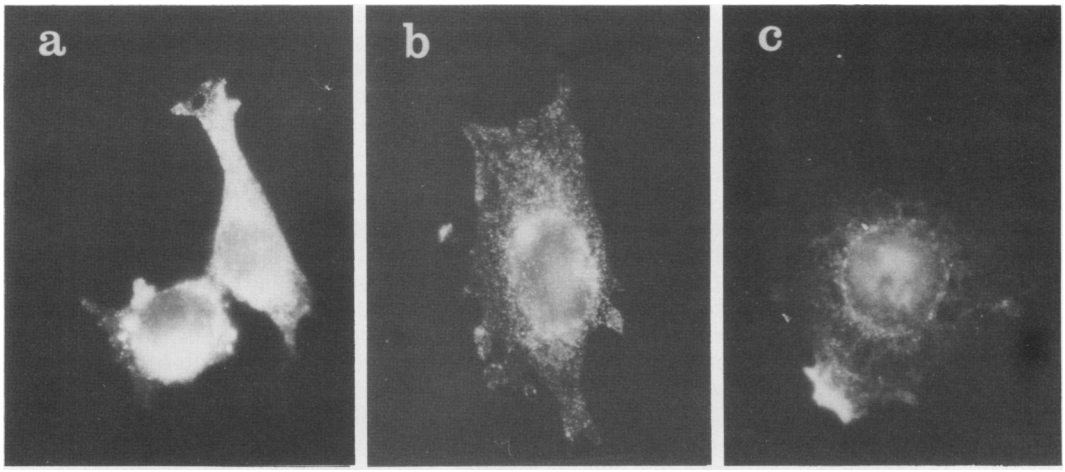

FIG. 7. *Papain treatment of living SV3T3 cells in a monolayer before analysis for T-Ag-related cell surface antigen. SV3T3 cells grown on cover slips in 3-cm petri dishes were washed with PBS and incubated with papain immobilized on carboxymethyl cellulose. Experimental conditions were chosen to remove the least possible number of cells from the culture dish by the action of papain. In this experiment only about 10% of the cells rounded up during a 20-min papain treatment. After 0 min (control, a), 10 min (b) or 20 min (c), the cover slips were removed from the petri dish, washed with PBS, fixed with formaldehyde, and processed for cell surface staining as described in the text. Rabbit anti-SDS T-serum at a 1:10 dilution in PBS was used as the first antibody followed by FITC-labeled sheep anti-rabbit immunoglobulin G. Exposure times were 30 s (a), 45 s (b) and 90 s (c). Magnification is 400-fold.*

sion or in ascites were analyzed, these cells were surface fluorescence positive without pretreatment. The very same cells, however, were surface fluorescence negative when analyzed as living cells in monolayer form on glass cover slips (R. Henning, J. Lange-Mutschler, and W. Deppert, submitted for publication). These data suggest that in contrast to SV40-transformed cells in suspension, surface T on adherent SV40-transformed cells is a cryptic antigen, which is not readily available for reaction with antibody. The different expression of surface T on adherent SV40-transformed cells and on SV40-transformed cells in suspension is not yet understood, but may be due to differing cell surface architecture.

In this study we extended our previous finding that T-Ag-related binding sites could also be exposed on adherent SV40-transformed monolayer cells when these cells were pretreated with formaldehyde before immunofluorescence analysis (Deppert and Henning, in press). Up to now, formaldehyde pretreatment of SV40-transformed cells seems to be the only means of allowing the in situ analysis of adherent cells for surface T by immunofluorescence microscopy. The mechanism by which formaldehyde exposes cryptic T-Ag binding sites on adherent cells is not yet understood, but one may assume that formaldehyde treatment might redistribute cell surface proteins or subsequently unmask T-Ag binding sites or both. Rearrangement of cell surface antigens as a result of formaldehyde treatment has been demonstrated by Flaherty and Zimmermann (12). In these experiments H-2^d and TL-antigens on the surface of mouse thymocytes were not simultaneously accessible to both anti-H-2^d- and anti-TL-antibodies on the surface of living mouse thymocytes. These antigens, however, became available for both antibodies after formaldehyde fixation.

Various controls have been employed to demonstrate that surface T, as detected by immunofluorescence microscopy on the surface of SV40-transformed cells after formaldehyde treatment, is indeed located on the outer surface of the plasma membrane. (i) Antiserum to actin, a major cytoplasmic protein, was negative on the surface of formaldehyde-fixed SV3T3 cells, indicating that antibody was not able to permeate the plasma membrane. (ii) The location of SV40 T-Ag-related molecules on the outer surface of the plasma membrane was shown more directly by analyzing the binding of *S. aureus* on the surface of formaldehyde-fixed SV3T3 cells precoated with rabbit anti-SDS T-serum. Since these bacteria are much larger than immunoglobulins, their penetration of the plasma membrane is unlikely. (iii) Finally, treatment of living SV40-transformed monolayer cells with carrier-bound papain removed the T-antigen-related binding sites from the surface so

that they were no longer available for anti-T-antibodies after formaldehyde fixation.

An important advantage of the formaldehyde fixation procedure developed in this study is that the fixed cells, after staining for surface T, could be permeabilized by treatment with organic solvents, thereby allowing the simultaneous analysis of these cells for both surface T and nuclear T-Ag. Since in these cells nuclear T-Ag could be detected with approximately the same immunofluorescence titer as in cells fixed by methanol–acetone treatment alone, the possibility of a simple "redistribution" of nuclear T-Ag (e.g., due to leakage) as a result of the formaldehyde treatment can be largely excluded. Furthermore, analysis of mixed cultures of 3T3 cells and SV3T3 cells by double staining immunofluorescence microscopy for both surface T and nuclear T-Ag indicated that possible exogenous T-Ag derived from dead cells did not precipitate unspecifically onto the surfaces of these cells during the fixation process.

Substantial support for the specificity of the cell surface staining obtained on formaldehyde-treated SV40-transformed cells with rabbit anti-SDS T-sera for SV40 T-Ag-related binding sites comes from the specificity criteria of these sera. (i) These sera are directed against purified SV40 T-Ag derived from SV40-transformed cells of different species origin and have been shown to specifically recognize SV40 T- and U-antigen (8, 9). (ii) These sera produce cell surface fluorescence only on SV40-transformed cells; all untransformed or non-SV40-transformed cells tested so far were fluorescence negative. (iii) Absorption of anti-T-antibodies from these sera not only significantly reduced the T- and U-antibody titers of these sera, but also abolished their ability to recognize T-Ag-related molecules on the surface of SV40-transformed cells. (iv) On *tsA* 28.3 cells (rat cells transformed by the SV40 *A* gene mutant *tsA* 28.3), these sera detected SV40 surface T only at the permissive growth temperature, i.e., when these cells synthesized the T-Ag polypeptide and expressed nuclear T-Ag. Upon shifting to the nonpermissive growth temperature, these cells became surface fluorescence negative simultaneously with the shutoff of the expression of nuclear T-Ag and the synthesis of the T-Ag polypeptide (W. Deppert, Virology, in press).

An interesting finding was that, as in our previous studies (Deppert and Henning, in press), SV40 T-Ag-related binding sites on the surface of formaldehyde-pretreated SV40-transformed cells could be detected by immunofluorescence microscopy with the rabbit anti-SDS T-sera only, not with SV40 tumor sera. In contrast, SV40-transformed monolayer cells put into suspension mechanically or by treatment with EDTA as well as SV40-transformed cells naturally growing in suspension were surface fluorescence positive both with rabbit anti-SDS T-sera and with SV40 tumor sera (21, 33; Henning et al., submitted for publication). Since the SV40 tumor sera used in this study had T- and U-antibody titers similar to those of the rabbit anti-SDS T-sera, the apparent lack of correlation between T- and U-antibody titers of these two types of antisera and their ability to recognize SV40 T-Ag-related binding sites on the cell surface of SV40-transformed cells might be explained by the following interpretations. (i) Formaldehyde treatment may have altered the antigenic binding sites of these proteins such that they are no longer recognized by the SV40 tumor sera even after exposure by formaldehyde. These denatured antigenic binding sites, however, would then be recognized by antisera prepared against SDS-denatured T-Ag. Although this possibility cannot be excluded by our experiments, we consider it unlikely, because nuclear T-Ag could be detected with tumor sera in cells fixed with formaldehyde and then permeabilized by additional treatment with methanol–acetone with approximately the same immunofluorescence titers as in cells fixed by methanol–acetone treatment alone. In accordance, Reiser et al. (29) describe that formaldehyde fixation of T-Ag loosely bound to SV40 chromatin did not alter the reactivity of tumor sera towards T-Ag. (ii) Alternatively, since surface T on adherent SV40-transformed monolayer cells is a cryptic antigen, one can imagine that the formaldehyde treatment employed exposed only T-Ag-related binding sites of surface T which are not or only weakly related to the T- and U-antigenic binding sites of nuclear T-Ag.

According to the latter possibility, a model for the expression of surface T on the surface of adherent SV40-transformed monolayer cells after formaldehyde treatment can be proposed which can be tested experimentally: surface T on adherent monolayer cells is a cryptic surface antigen, which can be exposed for a serological in situ analysis by alteration of the cell surface architecture, e.g., by treatment with formaldehyde. Formaldehyde treatment leads to a partial exposure of surface T, making available for reaction with antibody only those T-Ag-related binding sites which are different from the T- and U-antigenic binding sites of nuclear T-Ag as detected by SV40 tumor sera. According to this model, one would have to postulate that (i) surface T has a unique antigenic determinant(s) different from the T- or U-antigenic determinants of nuclear T-Ag, and (ii) further exposure of surface T on formaldehyde-treated SV40-

transformed monolayer cells should also expose T- and U-antigenic determinants on this molecule, thereby allowing its reaction with SV40 tumor sera, too. Both these postulates are indeed supported by several preliminary experimental data. Treatment of formaldehyde-fixed SV40-transformed monolayer cells with low doses of trypsin (1 μg/ml, 20°C, 3 min) resulted in the exposure of additional T-Ag-related binding sites on the surface of these cells, which then could also be recognized by SV40 tumor sera (unpublished data). These data strongly support the assumption that the failure of SV40 tumor sera to recognize surface T on formaldehyde-fixed SV40 transformed cells most probably is due not to a denaturing effect of the formaldehyde, but rather to the partial exposure of surface T, making available for reaction with antibody mainly a unique surface T determinant(s). Evidence for such a unique determinant of surface T, which is either absent or cryptic in nuclear T-Ag, was provided by Soule et al. Antisera prepared by these authors against surface membrane-associated SV40 T-Ag reacted only with the surface of living SV40-transformed cells in suspension, but not with nuclear T-Ag when tested on acetone-fixed cells (33). Similarly, we obtained antisera in BALB/c mice against intact formaldehyde-fixed mKSA cells, which were only very weakly positive in analyses for nuclear T-Ag, but specifically stained the surfaces of SV40-transformed cells (unpublished data).

If indeed formaldehyde-fixed, SV40-transformed monolayer cells expose mainly a unique antigenic determinant(s) of surface T, serological analysis of these cells might allow a further characterization of this determinant(s), thus providing a possible means to differentiate between nuclear T-Ag and surface T on a serological basis. Such a means should be very helpful in characterizing the molecular nature of SV40 surface T. Several recent experiments suggest that surface T may be a molecule identical to nuclear SV40 T-Ag or a closely related molecule with an identical electrophoretic migration behavior. (i) Soule and Butel (32) reported that a 94K protein indistinguishable from the 94K nuclear T-Ag could be immunoprecipitated from extracts of isolated membranes of SV40-transformed cells, and (ii) we found that lactoperoxidase-catalyzed ^{125}I iodination of surface proteins of SV40-transformed cells could label a 94K protein which was immunoprecipitable both with SV40 tumor sera and with rabbit anti-SDS T-sera after solubilization of the plasma membrane with Nonidet P-40 and was electrophoretically indistinguishable from the nuclear 94K T-Ag (Deppert and Henning, in press). In both sets of experiments, however, a possible contamination of "membrane" or "surface T" with nuclear T-Ag cannot be excluded. Reaction of surface T with an antibody specifically recognizing a possible unique surface T determinant(s) should help to circumvent these difficulties.

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